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Sex Differences In Insulin Resistance In GABAB1 Knock Out Mice

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Abstract

Aims: We have previously demonstrated that absence of functional GABA B receptors (GABABRs) disturbs glucose homeostasis in GABAB1KO mice. The aim of this work was to extend our studies of these alterations in GABAB1KO mice and investigate the sexual differences therein.

Main Methods: Male and female, GABAB1KO and WT mice were used. Glucose and insulin tolerance tests (GTT and ITT), and insulin and glucagon secretion tests (IST and GST) were performed. Blood glucose, serum insulin and hyperglycemic hormones were determined, and HOMA-IR calculated. Skeletal muscle insulin receptor β subunit (IRβ), insulin receptor substrates 1/2 (IRS1, IRS2) and hexokinase-II levels were determined by Western Blot. Skeletal muscle insulin sensitivity was assessed by *in vivo* insulin-induced Akt phosphorylation (Western Blot). Food intake and hypothalamic NPY mRNA expression (by qPCR) were also evaluated.

Key Findings: Fasted insulin and HOMA-IR were augmented in GABAB1KO males, with no alterations in females. Areas under the curve (AUC) for GTT and ITT were increased in GABAB1KO mice of both genders, indicating compromised insulin sensitivity. No genotype differences were observed in IST, GST or in IRβ, IRS1, IRS2 and hexokinase-II expression. Akt activation was severely impaired in GABAB1KO males while no alterations were observed in females. GABAB1KO mice showed increased food intake and NPY expression.

Significance: Glucose metabolism and energy balance disruptions were more pronounced in GABAB1KO males, which develop peripheral insulin resistance probably due to augmented insulin secretion. Metabolic alterations in females were milder and possibly due to previously described reproductive disorders, such as persistent estrus.

Keywords: insulin resistance; food intake; Akt activation; GABAB1KO mice

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Introduction

Type 2 diabetes (T2D) involves chronic dysregulation of glucose metabolism and impaired insulin sensitivity. More than 80% of patients progressing to T2D are hyperinsulunemic and insulin resistant (Pal 2009). In T2D, defects in insulin-stimulated glucose uptake in skeletal muscle are major factors inducing impaired glucose homeostasis, including alterations in protein expression, enzyme activation/deactivation or sensitivity to endogenous ligands.

GABA, the main inhibitory neurotransmitter in the brain, is found at high concentrations in Langerhans islets (Franklin and Wollheim 2004). Although a complete islet GABA system was demonstrated, the role of GABA in pancreatic physiology is less characterized. GABA inhibits high glucose-stimulated insulin secretion through GABAB receptors (GABABRs) in MIN6 cells and in rat/mouse islets (Bonaventura et al. 2012; Braun et al. 2004; Brice et al. 2002). We have recently described that GABAB agonists and antagonists alter glucose homeostasis in mice (Bonaventura et al. 2012). Previously we demonstrated that absence of functional GABABRs in GABABR knock-out mice (GABAB1KO) induced high pancreas insulin content and insulin resistance (Bonaventura et al. 2008).

Regarding insulin signaling, it binds to its receptor (IR), which is autophosphorylated and in turn phosphorylates IRS1 and IRS2. pIRS1/2 activate phosphoinsitol PI-3-kinase (PI3K) (Okada et al. 1994) which activates protein kinase B (PKB/Akt) (Franke et al. 1997). Akt is the major effector exerting the metabolic effects of insulin, including glucose transport, glycogen synthesis, fat deposition and protein synthesis. Loss of Akt signaling leads to glucose homeostasis impairment (Saltiel and Kahn 2001).

In addition to insulin, other hormones involved in blood glucose control are glucagon, growth hormone (GH) and corticosterone. GH increases plasma glucose by decreasing peripheral glucose uptake. An increased GABAergic tone inhibits GH secretion (Tuomisto and Mannisto 1985). Moreover, somatotropes express GABABRs (Mayerhofer et al. 2001) and stimulation of pituitary GABABRs increases GH secretion (Gamel-Didelon et al. 2002). Glucagon is secreted by α-cells in response to low blood glucose and its secretion is inhibited by insulin. Wendt et al. (Wendt et al. 2004) demonstrated that GABA released from β-cells inhibits glucagon release from α-cells in rat pancreas, confirming results in mice (Gilon et al. 1991). Ohers proposed that insulin sensitized α-cells to β-cell-secreted GABA (Xu et al. 2006). Cortisol increases blood

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glucose by inhibiting glucose uptake and utilization; it also stimulates appetite and changes fat metabolism (van Raalte et al. 2009). As GABA, through GABABRs, alters the corticotropic axis (Marques and Franci 2008), it could modulate the hyperglycemic effects of cortisol.

GABA is also considered an orexigenic stimulus (King 2006), and a role for central GABABRs controlling food intake has been suggested (Ebenezer and Prabhaker 2007). Neuropeptide Y (NPY), is expressed in the arcuate nucleus, and is a potent stimulant of food intake. Evidences suggest that the anorexigenic effects of insulin are exerted by inhibition of NPY in the arcuate, acting through GABAARs and GABABRs present in NPY neurons (Sato et al. 2005).

Therefore, here we evaluated how the lack of functional GABABRs affects different targets participating in blood glucose control in GABAB1KO mice and the sexual differences therein.

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Materials and Methods

Animals. GABAB1KO mice, generated in the BALB/C inbred strain (Schuler et al. 2001), were obtained by intercrossing heterozygous animals and the day of birth was recorded. Mice were genotyped by PCR analysis, as described previously (Catalano et al. 2005). Animals were fed ad libitum. All studies were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) that follows the NIH guidelines. Adult 2-3 month-old female and male WT and GABAB1KO mice were used. For each experimental design animals were age-matched littermates. Animals were sacrificed by decapitation in minimal conditions of stress.

Basal blood glucose titers and glucose tolerance test. Blood glucose was measured by a One touch® Ultra™ glucose meter (Lifescan, Scotland Ltd, strips were kindly donated by Johnson & Johnson, Argentina) from tail blood. For the glucose tolerance test (GTT) intraperitoneal (ip) glucose (3 g/kg body weight (BW) (Bonaventura et al. 2008)) was injected to overnight fasted mice (15-18 h) and blood glucose was evaluated at 0, 30, 60 and 75 minutes post glucose administration. Results were informed as Area under the curve (AUC).

Insulin determination and insulin secretion test (IST). Serum insulin was measured with an Ultrasensitive insulin mouse ELISA kit (Chrystalchem, Chicago, Il) at 0, 10, 20, 30 and 60 min after the ip glucose injection of 3 g/kg BW in mice fasted for 15-18 h in samples taken during the GTT. Results were informed as AUC.

HOMA index calculation. HOMA of insulin resistance (HOMA-IR) was calculated with basal blood glucose and basal insulin measured after overnight fasting, as previously decribed (Bonaventura et al. 2012). HOMA-IR= Fasting insulin (μU/ml) x Fasting glucose (mmol /L) / 22.5.

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Insulin tolerance test (ITT). Blood glucose was measured as above in 2-4 h fasted mice after 0, 10, 20, 30 and 60 min of an ip injection of 1 U/kg BW of porcine humanized insulin (a gift from Laboratorios Beta, Buenos Aires, Argentina). Results were informed as AUC.

Glucagon secretion test (GST). 3-4 h fasted animals were injected with insulin (1U/kg i.p.) and blood was collected from tail at 0 and 30 min post-injection (Zhou et al. 2004). Glucagon was determined by RIA (Glucagon RIA KIT, Millipore, MA) according to the manufacturer protocol. Results were informed as AUC.

Basal hyperglycemic serum hormones determinations. Glucagon was determined by RIA, as above. For corticosterone determination, serum samples were extracted with dichloromethane and corticosterone content determined by RIA, as previously described (Bonaventura et al. 2012; Repetto et al. 2010). Growth hormone (GH) was also determined by RIA, as previously described (Catalano et al. 2005).

In vivo peripheral tissue response to insulin. Fasted animals (2-4 hs) were anesthetized with avertin 2% (12 ml/kg i.p.). The abdominal cavity was opened, the portal vein exposed, and 2 U/kg of insulin was injected (in 0.5 ml saline) into the portal vein. At time points 0, 1 and 5 minutes post-injection, portions of skeletal muscle were excised and flash frozen in liquid N_2 and stored at -70ºC until used. Tissues were disrupted in 10 volumes of lysis buffer (1% SDS, NaCl, 10 mM EDTA, Tris.HCl) containing phosphatases and proteases inhibitors (LiCl, Na_3VO_4, PMSF, ZPCK, TAME, TPLC, β-glicerolphosphate) at 4ºC with a Polytron homogenizer, samples were centrifuged at 10000 rpm and pellets discarded. Supernatants were kept frozen until used.

Western Blot analysis. Western blot analysis for IRS1, IRS2, IRβ subunit, hexokinase-II, pAkt, Akt, and actin were performed in skeletal muscle homogenates of WT and $GABA_{B1}$KO of both genders. 50 μg of proteins and biotinylated molecular weight

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markers were subjected to 8% SDS-PAGE and proteins were transferred onto nitrocellulose membranes. Non specific protein binding to nitrocellulose was reduced by preincubating membranes with 5% non-fat milk in 0.5% Tween PBS (PBS-T). For IRS-1, IRS-2 and IRβ membranes were incubated over night (ON) at 4ºC with the first antibody diluted in PBS-T, 5% non-fat milk (Millipore, CA, IRS1, cat # 06-248: 1:500, IRS2, cat # 06-506: 1:500; IRβ, cat # 07-724: 1:125) followed by 2 hs incubating with HRP conjugated anti-rabbit (Vector, CA, cat # PI-1000: 1:3000) and anti-biotin (Cell Signalling, MA, cat # SP-3010: 1:4000) in PBS-T, 1% BSA, at room temperature (RT). For Hexokinase-II, membranes were incubated ON at 4ºC with first antibody generated in rabbit diluted in PBS-T, BSA 1% (Cell Signalling, cat # 2867: 1:500) followed by incubation with second antibody as described above. Membranes were stripped with stripping buffer (Tris-HCl 62,5 mM, SDS 2%, β-mercaptoethanol 100 mM, pH=6.7) and re-used for actin determination: membranes were incubated 1 h with mouse generated first antibody diluted in PBS-T, BSA 2% (Sigma, MI, cat # CP01: 1:5000) followed by incubation with HRP conjugated anti-mouse (Vector, CA, cat # PI-2000: 1:3000) and anti-biotin (Cell Signalling 1:4000) in PBS-T, 1% BSA, at RT.

For p-Akt, membranes were incubated ON at 4ºC with first antibody generated in rabbit diluted in PBS-T, BSA 5% (Cell Signalling, MA, cat # 9271: 1:1000) followed by incubation with second antibody as described above. Membranes were stripped, as described, and re-used for Akt; membranes were incubated 1 h with goat generated first antibody diluted in PBS-T, 1% BSA (Santa Cruz Biotechnology Inc, CA, cat # sc-1618: 1:500) followed by 1 h incubation with HRP conjugated anti-goat (Santa Cruz Biotechnology Inc, CA, cat # sc-2953: 1:4000) and anti-biotin (Cell Signalling 1:4000) in PBS-T, 1% BSA, at RT.

Detection was performed using an enhanced chemiluminescence Western Blot analysis

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system (Western Blotting Chemiluminescence Luminol Reagent, Santa Cruz Biotechnology) followed by image analysis with G-Box documentation system (Syngene, Unitek, BA).

Body weight and food intake. Body weight (BW) and food intake were monitored. Food intake was informed relative to body weight (g of food/g BW).

Expression of NPY in medial basal hypothalamus (MBH). To determine NPY expression by qRT-PCR, total RNA was isolated from homogenates of MBH, excised as previously described (Catalano et al. 2010). RNAs were obtained using TRIZOL reagent (Invitrogen, CA) according to the manufacturer's protocol and kept at -70ºC until used. The RNA concentration of all final preparations was calculated using the Qubit Quantitation Platform (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized from 2 µg of total RNA in the presence of 10 mM $MgCl_2$, 50mM Tris–HCl (pH 8.6), 75 mM KCl, 0.5 mM deoxy-NTPs, 1 mM DTT, 1 U/µl RnaseOUT (Invitrogen), 0.5 µg oligo(dT)15 primer (Biodynamics, BA), and 20 U of MMLV reverse transcriptase (Epicentre, WI). To ensure absence of genomic DNA the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA. For quantitative real-time PCR oligonucleotide primers sequence used were as follows: cyclophilin sense GTGGCAAGATCGAAGTGG, cyclophilin antisense TAAAAATCAGGCCTGTGG; NPY sense GATGCTAGGTAACAAGCGAATG, NPY antisense TCAGCCAGAATGCCCAAAC (Garcia-Tornadu et al. 2009). Quantitative measurements of cDNA were performed by kinetic PCR using SYBR® Green PCR Master Mix (Applied Biosystems, UK) according to the manufacturer's protocol, with an annealing step at 58ºC, 40 s. and an appropriate dilution of cDNA in a final volume of 13 µl.

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The accumulating DNA products were monitored by the ABI7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated based on the quality of dissociation curves, generated at the end of the PCR runs by ramping the temperature of the samples from 60°C to 95°C, meanwhile continuously collecting fluorescence data. Product purity was confirmed by 2% agarose gel electrophoresis. Each sample was analyzed in triplicate along with non-template controls to monitor contaminating DNA. The relative gene expression was normalized to that of cyclophilin housekeeping gene using the comparative method of ΔΔCt, described by the Livak & Schmittgen (Livak and Schmittgen 2001).

Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to a sample of wildtype male mice (calibrator sample).

Statistical analysis. All results are expressed as means ± SEM. Statistical analyses were performed with Statistica Six Sigma Edition. The differences between means were analyzed by one-way or two-way ANOVA, followed by Newman-Keuls test or Tukey HSD test for unequal N. For multiple determinations in the same animal, two–way ANOVA with repeated measures design was used, followed by the same post-hoc tests. $p<0.05$ was considered statistically significant.

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Results

Blood glucose and serum insulin. Fasted and non-fasted glycemia and insulinemia are summarized in table I. Fasted insulin was significantly higher in GABAB1KO male mice, (Two-way ANOVA, factors genotype and sex, interaction: $p<0.04$, GABAB1KO males significantly different from all the other groups, $p<0.01$ or less), but no differences in fasted glycemia were observed among groups. When calculating HOMA-IR, we observed a significant increase of this index in male GABAB1KO mice, pointing to the development of insulin-resistance in this group (Two-way ANOVA, factors genotype and sex, interaction: $p<0.03$, GABAB1KO males significantly different from all the other groups, $p<0.03$ or less). No HOMA-IR alterations were observed in females. In non-fasted mice only sex differences in blood glucose and serum insulin were observed (males > females for both parameters) without genotype differences.

Evaluation of blood glucose regulation. Since HOMA-IR was altered in male GABAB1KO mice, we performed functional tests to evaluate the clearance of serum glucose in a GTT, the glucose-induced insulin secretion in an IST, the insulin-induced glucagon release in a GST, and the peripheral insulin sensitivity in an ITT. The area under the curve (AUC) for each of these dynamic tests is shown in Fig. 1. The GTT was altered in GABAB1KO mice (Fig. 1A), showing an increase of AUC both in male and females with respect to their WT controls, in addition to a sex difference (males > females) (Two-way ANOVA, interaction: ns, factor genotype: $p<0.01$, factor sex: $p<0.02$). These results indicate that the clearance of glucose form blood is impaired in GABAB1KO mice. To define whether these alterations are due to reduced insulin secretion or to diminished insulin sensitivity, we performed the IST and ITT. The IST AUC was not altered in GABAB1KO mice with regard to their same sex controls; a statistically significant sex differences was observed in the IST AUCs, males > females, in both genotypes (Fig. 1B).

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However, insulin sensitivity was diminished in GABAB1KO mice, since AUCs of ITTs were increased in this genotype (Fig. 1C); in addition a gender difference was also observed (males > females) (Two-way ANOVA, interaction: ns, factor genotype: $p<0.01$, factor sex: $p<0.01$). This result indicates that, in response to the same insulin stimulus, depuration of glucose from blood is impaired in GABAB1KO mice. Finally, we found no genotype alterations in the GST in response to insulin although GST AUCs were increased in females with regard to males (Fig. 1D) (Two-way ANOVA, interaction: ns, factor genotype: ns, factor sex: $p<0.01$), suggesting that alterations in glucagon secretion were not involved in the observed phenotype in GABAB1KO mice.

Evaluation of hyperglycemic hormones. When analyzing other hormones involved in the control of glucose homeostasis, we found diminished serum corticosterone levels in GABAB1KO respect to WT females (Fig. 2A). In addition, in GABAB1KO mice the expected sex difference observed in WTs (females > males) had disappeared. GH and glucagon showed neither sex nor genotype differences (Fig. 2B and C, respectively).

Expression of proteins involved in the insulin signaling pathway in peripheral tissue. Since dynamic tests showed an alteration of peripheral insulin sensitivity, we evaluated the insulin signaling pathway on skeletal muscle, the main tissue involved in glucose depuration. We first evaluated the expression of main proteins involved on this cascade, finding no differences in basal expression of IRS1, IR2, IRβ or hexoquinase (Fig. 3A, 3B, 3C and 3D, respectively).

Insulin-induced Akt phophorylation. Activation of Akt is the key event responsible for the increase in insulin-induced glucose uptake by skeletal muscle. Activation of Akt (Fig. 4) showed no genotype alterations in females (Two way ANOVA, interaction: ns, factor genotype: ns, factor time: $p<0.01$, time 0 min: significantly different from 1 and 5 min, $p<0.01$). In contrast, in GABAB1KO males phosphorylation of Akt was markedly

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impaired at 5 minutes post insulin administration (Two way ANOVA, interaction: $p<0.04$, WT males at 5 min: significantly different from 0 and 1min, $p<0.02$ and also significantly different from GABAB1KO males at 5 min, $p<0.01$).

Body weight, food intake and NPY expression. Figure 5 shows food intake relative to BW and expression of NPY mRNA of animals in this study. GABAB1KO mice show increased food intake compared to WTs (Fig. 5A) (Two way ANOVA, factors genotype and sex, interaction: ns, factor genotype: $p<0.05$). Despite this increase, no genotype differences were found in body weight (not shown), as previously described (Catalano et al. 2005). Interestingly, expression of NPY mRNA was also altered in GABAB1KO mice, being significantly higher than in WT mice (Fig. 5B) (Two way ANOVA, factors genotype and sex, interaction: ns, factor genotype: $p<0.05$).

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Discusion

The worldwide rise in the incidence of obesity, metabolic syndrome and diabetes, and the increasing costs in the management of these diseases and their complications require a comprehensive knowledge of the factors regulating glucose homeostasis in order to advance in the treatment of these disorders.

GABA is present at high concentrations in Langerhans islets and its participation in islet physiology is multifactorial, including actions in cell metabolism and viability (Ligon et al. 2007; Soltani et al. 2011; Sorenson et al. 1991; Winnock et al. 2002) and species-specific autocrine and paracrine effects on hormone release (Bonaventura et al. 2008; Braun et al. 2004; Braun et al. 2010; Brice et al. 2002; Robbins et al. 1981; Soltani et al. 2011; Wendt et al. 2004; Xu et al. 2006).

We have demonstrated that GABAB agonists and antagonists, administered both acutely or chronically, disturb the regulation of glucose homeostasis in BALB/C mice (Bonaventura et al. 2012). In addition, we reported that the absence of functional GABABRs in male GABAB1KO mice revealed signs of insulin resistance and increased pancreas insulin content (Bonaventura et al. 2008). As sex differences in the incidence of diabetes has been reported in various animal models such as NOD mice (Rosmalen et al. 2001) and STZ-induced diabetes (Vital et al. 2006) here we performed an in depth study of the alterations in the glucose homeostasis regulation in GABAB1KO mice comparatively in both genders.

There were no genotypes differences in blood glucose or serum insulin in animals in non-fasting state. However, fasted insulin was significantly increased in GABAB1KO males, while maintaining euglycemia. The presence of hyperinsulinemia accompanied by euglycemia suggests a reduced sensitivity to insulin of peripheral tissues. This phenomenon is further put into evidence by HOMA-IR index, which was significantly

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increased in GABAB1KO males, as also reported in another model of insulin resistance (Maiztegui et al. 2009). Supporting this hypothesis, GABAB1KO males also showed impaired ITTs, indicating decreased glucose depuration. Putting these results together, it can be inferred that GABAB1KO males developed an impaired glucose tolerance derived from diminished insulin sensitivity in peripheral tissue. The sustained, increased, fasted insulin secretion observed in GABAB1KO males, due to the absence of functional GABABRs, which is in agreement with increased pancreas insulin content described previously (Bonaventura et al. 2008), may induce, over time, insulin resistance in peripheral tissues, as described in functional insulinomas, in which alterations in insulin receptor splice variants, signaling, and binding have been demonstrated (Nankervis et al. 1985; Sbraccia et al. 1996; Skrha et al. 1996). In this line, in mouse C2C12 muscle cells constant insulin stimulus triggered insulin resistance (Kumar and Dey 2003; Rui et al. 2001), which was evidenced by decreased expression and/or phosphorylation of different elements of the insulin signaling cascade.

Given the evidence that GABAB1KO male mice develop insulin resistance spontaneously, we evaluated whether any elements of the insulin signaling cascade were altered in these animals, especially in skeletal muscle, the main tissue involved in the insulin-stimulated-glucose uptake from blood. No differences in the expression of IRβ, IRS1/2 and hexokinase-II were observed in our model. Interestingly, several studies have shown that the number and function of the insulin receptors are normal or slightly reduced in patients and animal models of insulin resistance, and that this is not sufficient to explain the marked reduction in insulin function, thus suggesting that major alterations are downstream the receptor (Draznin 2006). Therefore, we then assessed the activation of Akt, the main effector in the cascade that leads to membrane translocation of glucose transporter in muscle (GLUT-4). We observed that the insulin-induced Akt

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phosphorylation was profoundly impaired in GABAB1KO males compared to their WT controls. This result is in agreement with other authors who report a decrease in insulin-stimulated Akt phosphorylation in skeletal muscle of mice spontaneously developing glucose intolerance and insulin resistance (Oh et al. 2008). Thus, this severely diminished activation of Akt could justify the insulin resistance observed in GABAB1KO males. Conversely, no alterations were found in hyperglycemic hormones such as glucagon, GH and corticosterone between genotypes in male mice.

In GABAB1KO females, glycemia and serum insulin were normal in both the fasting and non-fasting states. Furthermore, we found no alterations in the HOMA-IR index, as opposed to GABAB1KO males. However, results from ITTs and GTTs are somewhat puzzling, since they are compatible with diminished insulin sensitivity of peripheral tissues. The hyperglycemic hormones studied showed diverse results in females; GH and glucagon showed no differences between genotypes, but corticosterone was altered in GABAB1KOs. Corticosterone was augmented in WT females compared to males, in agreement with bibliography (Bastida et al. 2007; Vasan et al. 2004). In contrast GABAB1KO females presented significantly lower serum corticosterone, thus losing the expected gender difference. Anyway, these results cannot justify the genotype differences observed in glucose disposal. One possible explanation of the discrepancy in GTTs and ITTs with respect to glycemia and serum insulin is the fact that GABAB1KO females show persistent estrus (Catalano et al. 2010), an estrous cycle stage characterized by low serum estradiol (Neill 1980); this can also explain the differences observed in corticosterone, given that estrus is also characterized by lower levels of this glucocorticoid (Atkinson and Waddell 1997). Regarding the effects of estradiol on insulin resistance, Bruns and Kemnitz (Bruns and Kemnitz 2004) described that women in the luteal phase, characterized by lower serum estradiol, have decreased

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insulin sensitivity during a GTT. Additionally, the authors also described diminished insulin sensitivity and disposition during a GTT in the luteal phase of female monkeys. Hence, we suggest that GABAB1KO females do not have primary insulin resistance but the diminished sensitivity observed is secondary to alterations in their reproductive axis, which we have previously described (Catalano et al. 2005; Catalano et al. 2010). The protective effect of estradiol against the development of insulin-resistance and diabetes mellitus is widely discussed in bibliography (Prasannarong et al. 2012; Sakata et al. 2010). In this regard we can postulate that, even in estrus, females, both GABAB1KO and WT, are less prone to develop these conditions than males, and this can explain the gender differences we observed in glucose tolerance and insulin sensitivity. This hypothesis is also strongly supported by the lack of modification in the insulin signaling pathway in skeletal muscle, although alterations in other insulin sensitive tissues such as adipose or liver may be involved in the weak disturbances observed in GABAB1KO females.

So far, we conclude that male GABAB1KO mice present a clear insulin-resistance syndrome probably developed as a consequence of persistent insulin secretion, while female GABAB1KO mice present some features of diminished insulin sensitivity, which are not due to augmented insulin, and probably due to alterations in the reproductive axis.

The mechanisms involved in the regulation of energy metabolism are complex and varied. GABA modulates the expression/release of several factors that modulate food intake and its effect varies in different hypothalamic areas and also depends on the GABA receptor involved (Ebenezer 2012; Kamatchi and Rathanaswami 2012; Perdona' et al. 2011; Stanley et al. 2011). NPY is among the most studied orexigenic factors (Kalra and Kalra 2004; Mercer et al. 2011; van den Pol 2003) and is also regulated by GABA (Sato et al. 2005). We found an increase in food intake in GABAB1KO animals compared to WTs. This increase, however, was not accompanied by weight gain,

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indicating that the energy balance might be altered in these mice possibly due to increased locomotor activity described previously (Schuler et al. 2001). Based on these results, we evaluated the expression of NPY in MBH finding a significant increase in GABAB1KO mice of both genders. Some studies have postulated that GABA acting on GABAB receptors decreases food intake (Perdona' et al. 2011). Therefore, lack of functional GABAB receptors may have induced the increase in food intake. Others have determined that GABA has orexigenic effects in the ventromedial hypothalamus (Perdona' et al. 2011). In this regard, several authors described that muscimol, given ICV or directly in ventromedial hypothalamus or paraventricular nucleus, induced increases in food intake (Choquette et al. 2009; Jonaidi et al. 2012; Kalra and Kalra 2004; King 2006). Lack of RGABAB expression may also cause changes in other components of the GABA system; our laboratory has described an increase in GAD67 mRNA in MBH (Catalano et al. 2010), so an increase in GABA content could be expected and this could also justify the increase in food intake. Moreover, NPY neurons in the arcuate nucleus that monitor serum glucose levels have GABAergic innervation, and GABA, through GABAB and A receptors, inhibits neuron activity attenuating the orexigenic effects of NPY (Muroya et al. 2005). Therefore lack of GABAB receptors could have potentiated the effect of NPY on food intake. In addition, Sato et al also suggest that insulin increased GABA release, which in turn acted on GABABRs to suppress NPY gene expression (Sato et al. 2005). These results are in agreement with our findings since the lack of inhibition due to GABABR absence may have lead to an increase in NPY expression and the consequent increase in food intake.

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Conclusion

In sum, the absence of functional GABAB receptors induce multiple, sex-dependent metabolic alterations put into evidence in the GABAB1KO mouse. GABAB1KO males share many characteristics with the insulin resistance syndrome, including increased fasted insulin and HOMA-IR, dysfunctional GTTs and ITTs and peripheral desensitization to insulin-stimulated AKT phophorylation, as well as increased food intake, although these parameters do not lead to weight increase. The female phenotype is less severe showing mild food intake increase and glucose intolerance, this last condition being possibly secondary to reproductive disruptions. These observations further emphasize the importance of GABA and its GABAB receptors in metabolic regulation and reinforce the gender differences often observed in related diseases.

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Conflict of interest:

Authors declare that there are no conflicts of interest involved in this manuscript.

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Figure legends

Figure 1. A) Area under the curve (AUC) for the glucose tolerance test (AUC GTT). Two-way ANOVA: interaction: NS, factor sex (#): $p<0.02$, genotype (*): $p<0.002$. B) AUC for the insulin secretion test (AUC IST). Two-way ANOVA: interaction: NS, factor sex: $p<0.001$, genotype: NS. C: AUC for insulin tolerance test (AUC ITT), Two-way ANOVA: interaction: NS, factor sex: $p<0.01$, factor genotype: $p<0.01$. D) AUC for the glucagon secretion test (AUC GST). Two-way ANOVA: interaction: NS; factor sex: $p<0.04$, factor genotype: NS.

Figure 2. Basal serum levels of hyperglycemic hormones. A) GH: two-way ANOVA: NS; B) Glucagon: two-way ANOVA: NS; C) Corticosterone: two-way ANOVA: interaction, $p<0.01$, *: WT females significantly different form all groups, $p<0.05$ or less.

Figure 3. Expression of proteins involved in the insulin signaling pathway in skeletal muscle. A) IRS-1, B) IRS-2, C) IRβ and D) Hexokinase. In all cases two-way ANOVA: NS.

Figure 4. Insulin-induced Akt activation in skeletal muscle. Females: two-way ANOVA: interaction: NS, factor genotype: NS, factor time: $p<0.01$, a: 1 and 5 min significantly different from 0 min, $p<0.01$. Males: two-way ANOVA: interaction: $p<0.04$, *: WT males at 5 min significantly different from 0 and 1 min, $p<0.02$; a: WT males at 5 min significantly different from GABAB1KO males at 5 min, $p<0.01$

Figure 5. A) Food Intake (g/BW(g)). Two-way ANOVA: interaction: NS, factor sex: NS, factor genotype: $p<0.03$, *: GABAB1KO different from WT. B) Hypothalamic NPY mRNA expression. Two-way ANOVA: interaction: NS, factor sex: NS, factor genotype: $p<0.04$, *: KO different from WT.

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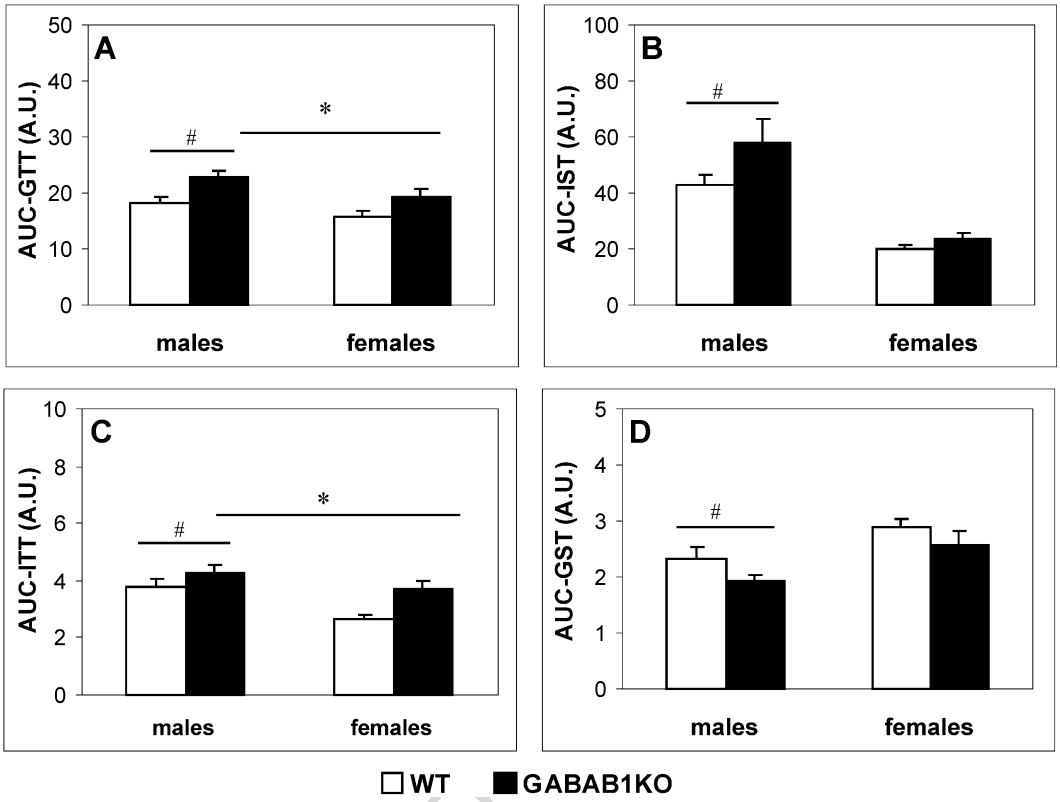

Fig. 1

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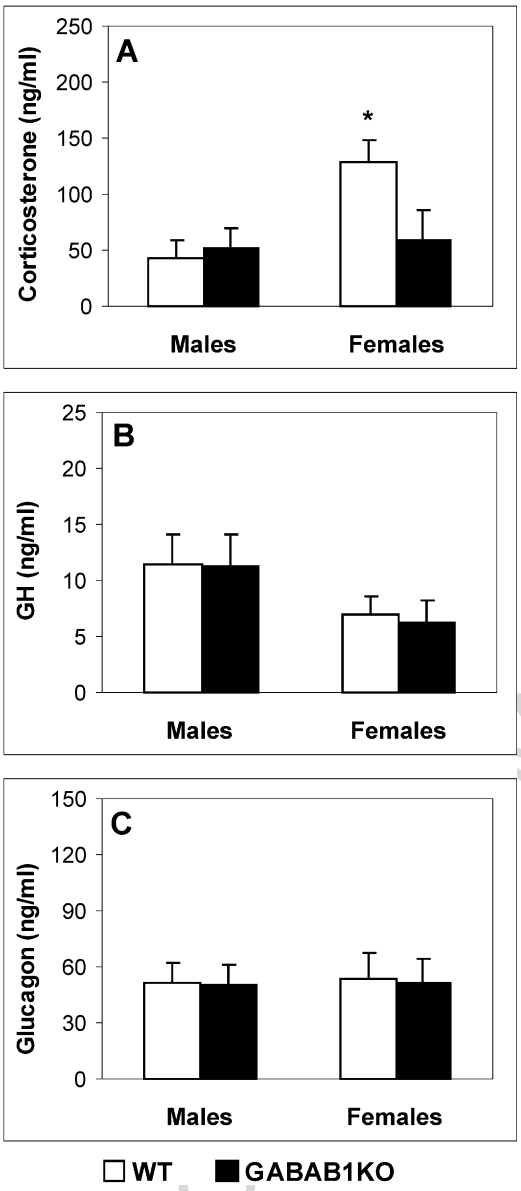

Fig. 2

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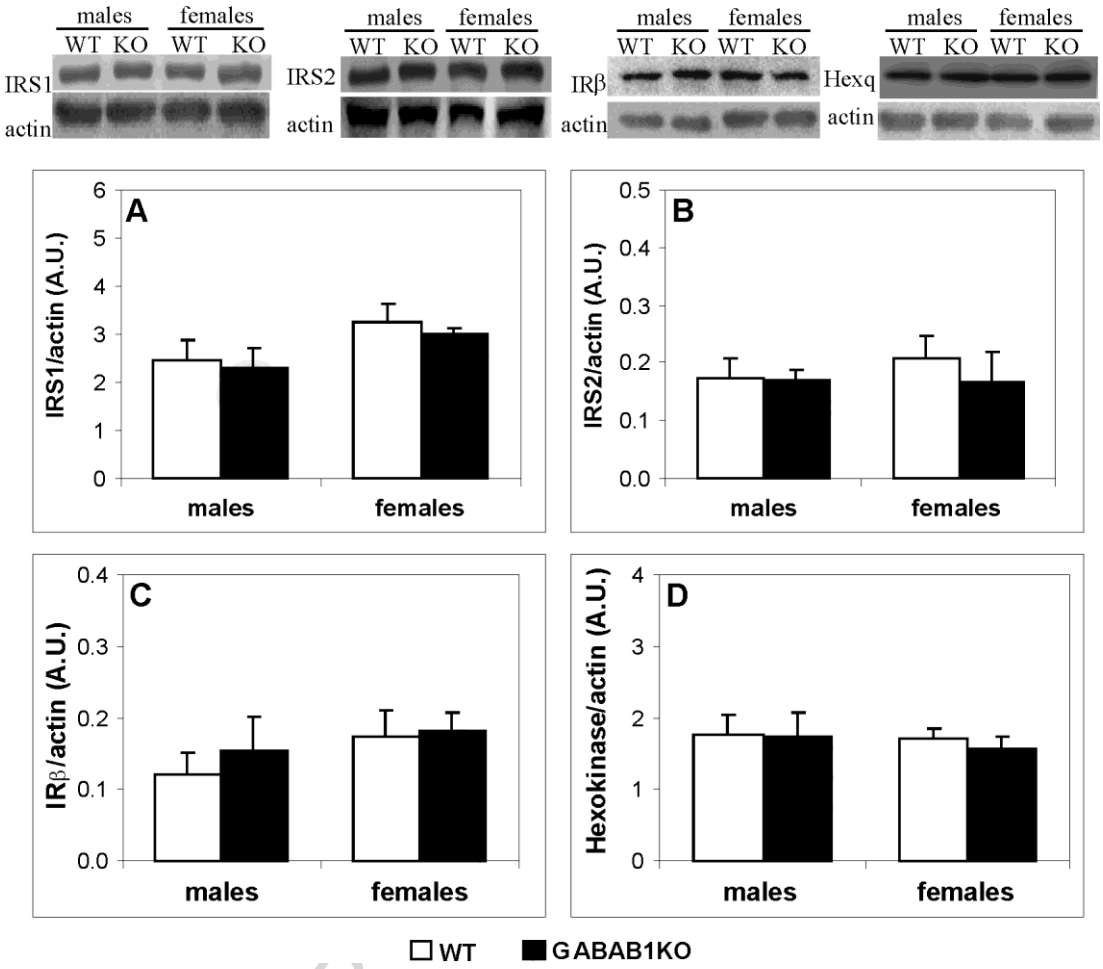

Fig. 3

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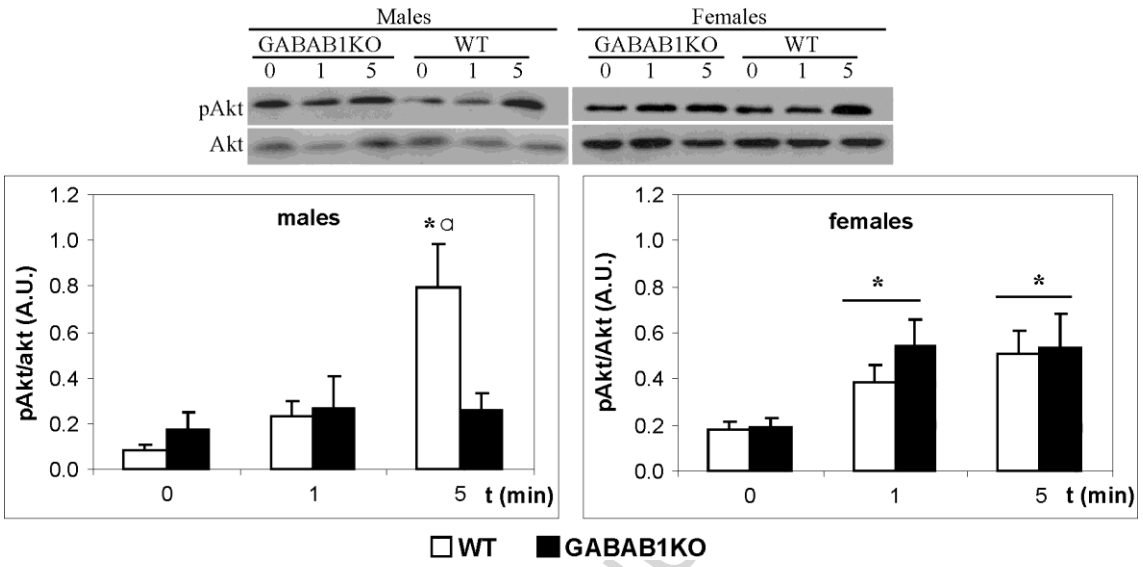

Fig. 4

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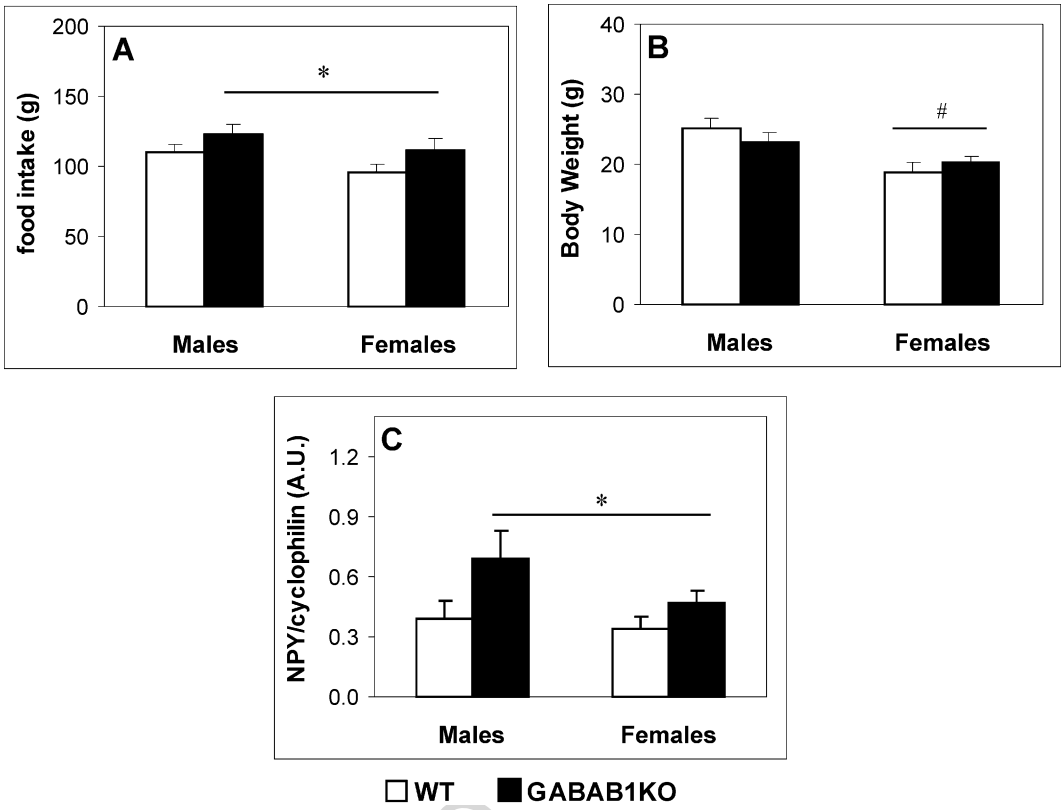

Fig. 5

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Table I. Basal blood glucose, serum insulin and HOMA-IR index

	Males		Females	
	WT	GABAB1KO	WT	GABAB1KO
Non-fasted Blood Glucose (mg/dl)	129 ± 4#	123 ± 5#	103 ± 4	104 ± 6
Fasted Blood Glucose (mg/dl)	78 ±3	89 ± 9	82 ± 4	86 ± 4
Non-fasted Serum Insulin (ng/ml)	0.40 ± 0.08#	0.41 ± 0.09#	0.27 ± 0.05	0.25 ± 0.03
Fasted Insulin (ng/ml)	0.18 ± 0.03	0.45 ± 0.11*	0.14 ± 0.02	0.14 ± 0.02
HOMA-IR	0.84 ± 0.16	2.24 ± 0.48*	0.63 ±0.07	0.59 ± 0.08

Values are expressed as Media ± SEM. Non-fasted blood glucose: two-way ANOVA: interaction: NS, factor sex: #: $p<0.001$, factor genotype: NS. Non-fasted serum insulin: two-way ANOVA: interaction: NS, factor sex: #: $p<0.05$, factor genotype: NS. Fasted serum insulin: two-way ANOVA: interaction: $p<0.04$, *: GABAB1KO males different from the other groups, $p<0.01$ or less. HOMA-IR: two-way ANOVA: interaction: $p<0.03$, *: GABAB1KO males different from the other groups, $p<0.03$ or less. Number of animals: 15-20 animals each group.